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APPLICATION OF FLUORESCENT ANTIBODIES IN EXAMINING THE STRUCTURE OF THE EYEBALL

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The principle of marked antibodies was developed by Coons [8, 9] on the basis of the observation that the molecules of antibodies can be chemically connected with simple chemical compounds or dyes without disturbing the reactiveness peculiar to antibodies.

The first data on the discovery of antigen material with the aid of antibodies marked with fluorescence appeared in 1942 [10], and the further improvement of this method occurred in 1950 [11]*. Since then many works have appeared on the application of this method in biology and medicine. They concern research on contagious factors introduced into the organism by specific foreign antigens, tissue antigens and by the production of antibodies and γ-globulin through the cells of the organism or in the sphere of pathology with special consideration of diseases of still undetermined etiology -- however, a special discussion of them [2, 4, 18] would go beyond the framework of this work.

I will limit myself to a discussion of the eyeball with consideration given to research on antigen structures, the local production of antibodies and on the identification of microorganisms introduced under experimental conditions and during illnesses.

*I direct the attention of readers who are interested in the technical details of fluorescent antibodies to the work by Henryk Matej, "Fluorescent Antibodies," Postepy Higieny i Medycyny Doswiadczalnej (Progress of Hygiene and Experimental Medicine), 1961, 15, 463.

12

In experiments on the eyeballs of a one week old mouse embryo Clayton [5] used γ-globulin fractions of serum immune to the lenses and muscles. The author proceeded from the assumption that the subjection of many antigens with varied peculiarities and of sera combined with various dyes to simultaneous examination makes it possible to trace the disintegration of antigens in various stages of individual development. She used three fluorochromes, namely: of a yellowish green color (fluorescein), red (benzaldehyde-b nitro-2-sodium-azotoate) and yellow (1-dimethyloamino-5-sulfonylchloride-naphtalene). In examining small portions of the eyeball the author observed a bright red coloring of the lens, a pale-pink coloring of the ciliary corpuscle and of the retina, pale-green choroid and a green coloring of the internal muscles and Zinn's ligament. The processes of the ciliary corpuscle, which in Amphibia is capable of regenerating the lens, according to Clayton contain certain quantities of lens crystallin or albuminous bodies of the sclera. On the basis of obtained results, she confirms that the choroid and Zinn's ligament possess antigens similar or identical with the antigens of the muscles. Clayton also observed that the serum against the lens reacted with the cerebral tissue in the early stages of the embryogenesis of the mouse, while this reaction was expressed somewhat stronger than in the fibers of the eye. A cross reaction also took place between the fluorescent serum against the lens and the cells of the epithelium; it was expressed more strongly in the full-grown animal.

It should be mentioned that the results of Clayton's research found confirmation during the application of the method of antibodies marked with an iodine isotope [6], in which the quantitative data was obtained on the histochemical localization of antigens in the eyeball.

Maisel [17] also reported on the appearance of lens antigens in other tissues of the organism on the basis of experiments conducted according to the method of dual precipitation in the agar gel. The results of these experiments, to a significant degree, coincide with the observations Clayton made with the help of fluorescent antibodies.

In the research on the antigen structures of the tissues of the eyeball Roberts [22] used fluorescent globulin from rabbit serum containing antibodies against nephritic glomerules of a rat. This globulin reacted in its own way with the basal membranes of not only the kidneys, but also of other tissues. In using a special technique of removing the thin fibers from the eyeball of a rat, Roberts dyed them with fluorescent serum against the nephritic glomerules of the rat. In examining the peculiarities of the various dyed portions of the eyeball Roberts encountered considerable difficulties connected with the phenomenon of

autofluorescence. In the preparations from the eyeball of a rat the serum against nephritic glomerules reacted in a peculiar manner with the lens capsule, however, it was not possible to distinguish the real capsule from Zinn's ligament. After completion of the reaction, fluorescence did not occur in the area of the lens, which confirmed the peculiarity of dyeing. The fluorescent membrane was clearly visible under the endotheliem of the ciliary corpuscle and in the ciliary corpuscle. A membrane located between the endothelium and the shell was observed in the iris and around the constrictor of the iris a dark coloring in the dyed fibers was confirmed by fluorescent serum, which would lead one to assume that this phenomenon appears in the presence of antigens peculiar to it. In the area of the cornea a strong autofluorescence appears, which made it impossible to give an interpretation of the results. In examining the the fibers of the conjunctiva Roberts confirmed a thin membrane visible below the epithelium. The capillaries exhibited their own coloring. In the area of the rear section of the eyeball the author confirmed a strange coloration of the sclera. The capillaries in the retina and in the optic nerve exhibited their own coloration.

On the basis of the results obtained Roberts confirms that the peculiar coloration of the basal membranes of the ciliary corpuscle, the iris and of the capillaries of the retina, the optic nerve and of the conjunctiva, one could assume that they possess common antigens with the basal membrane of the nephritic glomerules.

Germouth and co-workers [13] conducted immunohistological examinations on the reaction of a fluorescent antigen with antibodies in a cornea devoid of blood vessels. The result of the work of these authors was the commonly accepted observation that in a state of hypersensitivity a local reaction of the tissue results from damage to the capillaries which take the place of the reaction of the antigen with the antibodies. The fluorescent antigens were cattle albumen and γ-globulin marked with fluorescein. Albino rabbits were made sensitive subcutaneously by albumen and globulin fraction over a period of three months, and were then injected with fluorescent antigens through the cornea. The injection of homologous antigen into the veinless cornea of sensitized animals led to the development of an annular opacity located between the center and the edge of the cornea. This opacity consisted of lines composed of a dark, amorphous, eosinophilic material located in the matrix of the swollen collagenous fibers, between which the multicellular leukocytes are located. With the help of fluorescent antigen it was shown that the line of damage in the cornea coexisted with the precipitation of the antigen with the antibodies originating from the vessels located at the edge of the cornea. After a certain time of observation

the precipitates underwent a sucking-in process, the annular opacity, however, underwent hypertrophy through the blood vessels encompassed by plasma cells.

Morawiecki and Brzosko [19] observed that during the diffusion of the antigens and antibodies in the cornea of the rabbit a phenomenon occurred similar to the precipitation in the agar gell. After the introduction of horse serum into the cornea on the one hand and of a homologous antibody on the other, strips composed of a concentration of leukocytes appeared in the cornea after 24 hours between both places of injection. In order to obtain a state of hypersensitivity Morawiecki and Brzosko injected fluorescent horse serum in the cornea over a period of three weeks. In turn they injected a dose into the cornea which led to an appearance of precipitation rings around the point of injection, in the area of which they confirmed the presence of a strongly fluorescent precipitate.

An interesting contribution in the tests on local production of antibodies in the eyeball are the penetrating observations of Witmer [26] made in connection with the experimental infection of the eyeball of a rabbit with a Leptospira pomona suspension introduced into the aqueous humor. The production of antibodies, as Witmer confirmed, took place in the eyeball and was connected with the depositing of plasma cells and lymphocytes in the uvea. The titer of antibodies contained in the -globulin fraction of chamber fluid in the local infection was greater than the analogous titer of serum antibodies. The activeness of antibodies in the eyeball was closely connected with the degree of depositing in the chamber uvea.

The results of the experimental tests, which were obtained by Witmer [27] after pre-chamber and pre-vitreous introduction of chicken albumin, confirmed the assumption that the antibodies in the uvea are contained in the plasma cells, while, on the other hand, the mesenchymal cells of the iris and of the ciliary corpuscle as well as the cells of the endothelium and the cells of the cornea did not contain antibodies. In some cases the antibodies were adsorbed in Descemet's membrane and in the fibers of the cornea. Additional plasma cells in the area of the iris and the ciliary corpuscle were found in the eyeballs which showed a high titer of antibodies in the chamber fluid. After injection of chicken albumin the production of antibodies was not limited just to the uvea changed by inflammation, but a simultaneous systematic production of antibodies occurred, after which a high titer of antibodies appeared in the blood serum. A deposit consisting of cells containing antibodies was confirmed around the caterpillar hairs which were introduced into the front chamber of the eye as a local irritant.

Using fluorescent antibodies Coleman and Becker [7] conducted experiments for the purpose of localizing γ-globulin in the human eyeball, which showed changes on the basis of diabetes. For this purpose, they used fluorescent antiglobulin serum, with which they dyed the fibers of the eyeball for observing changes in the basal membrances of the ciliary corpuscle and the iris. In the examined eyeballs the fluorescent antiglobulin serum colored the minute aneurisms found in the retina. Fluorescence was also observed in the basal membranes of the ciliary corpuscle.

In examinations on the inflammation mechanism of the uvea, much significance is ascribed to the action of bacteria and their toxins [14], because many authors have searched for streptococcus and staphylococcus in the chamber fluid by using seeds on special bases for this purpose [1, 3]. The low percentage of detection of pathogenic microorganisms in cases of inflamed uvea, according to Offret [20] results from the peculiarities of the chamber fluid which are harmful to bacteria. The role of toxins can be attested to by the results of tests conducted by Ogielski and co-workers [12], who applied the dyed reaction of Koloblocki for detecting bacterial toxins in the chamber fluid of animals with experimentally induced inflammation of the uvea. According to the authors, the experiment by Koloblocki can find application as a test case of the appearance of the bacterial-toxic factor in inflammations of the anterior segment of the uvea.

The applicati n of fluorescent antibodies in the experiments on the inflammation mechanism of the uvea would have found justification for they make it possible to detect microorganisms in situ. Lipnicki and Reiss [16] conducted attempts to detect Str. pyogenes in the fluid of the anterior chamber of the eye of experimentally infected rats, using serum which precipitates "great sugar" (wielkocukier) C of streptococci from the group A, which was marked with isothiocyanate of fluorescein. The chamber fluid was taken for testing 3, 4, 8 and 25 days after infection and direct preparations were made up which were dyed according to the method of Gram and Manson and by fluorescent serum. At the same time, a sclerotic culture and cultures on artificial bases were made from the collected exudation. In order to exhibit the peculiarities of dyeing by fluorescent serum, a retarding reaction of the fluorescence was carried out. On the third day of the development of the inflammation process of the eyeball, striptococci were detected most easily in the direct preparations dyed according to the method of Gram or Manson, but with certain difficulties -- in preparations dyed with fluorescent serum. On the fourth day, during maximumly expressed changes ab externo, streptococci were easily detected by all the methods discussed, on the eighth day the same results were achieved as on the

third day, while on the other hand no streptococci were detected on the 28th day. Thanks to the 2-hour multiplication of streptococci in the sclerotic culture, the identification of Str. pyogenes with the aid of fluorescent serum became completely attainable. In the fluorescent microscope streptococci showed a stronger circumferential fluorescence (Figure 1).

The retarding reaction of fluorescence exhibited the peculiarity of dyeing used for the infection of a strain of Str. pyogenes in a sclerotic culture made up of the exudation collected from the anterior chamber of the eye. According to the authors the sclerotic culture connected with the method of immunofluorescence creates certain possibilities for identifying pathogenic microorganisms in the chamber fluid.

The application of fluorescent antibodies also extends to the detection of antigen virus material in the infected cells. Also in use is the direct detection of virus antigens by a homologous fluorescent antiserum as well as the method of the intermediary given for the first time by Weller and Coons [25]. They acted on the cells of the fiber culture infected with viruses by their own antivirus serum and in turn acted by fluorescent anti-γ-globulin. It precipitated in places, where the virus antigen joined with its own antibodies producing fluorescence.

Figure 1. Preparation from the sclerotic culture of the chamber fluid, collected from the eyeball of a rat infected with a Str. pyogenes suspension. Dyeing by fluorescent serum for group A streptococci (enlargement 10 X 90).

For the purpose of confirming the etiology of inflammations of the cornea and sometimes of the uvea caused by viruses of cold sores, Witmer [28] conducted experimental

tests using the direct method of fluorescent antibodies, on the basis of a technique worked out by Kaufman [15] and applied in the diagnostics of keratitis herpetica. After obtaining the marked serum made resistant against the virus of cold sores, Witmer applied it to the examinations on the fiber culture of cells of epithelial cancer which were infected with the mentioned viruses. Forty-eight hours after the infection, cytopathologic changes, which depend on the rounding of the outlines of cells and on the appearance of damages, occurred. With the help of fluorescent serum he confirmed that the zone of fluorescence was especially clear around the nucleus of cells infected with viruses. In the well developed areas of virus infection it was impossible to confirm a clear cell structure.

While examining the epithelium of the cornea, which was conducted in cases of fresh and chronic cold sore inflammations with an established clinical diagnosis, Witmer observed, in addition to the barely visible cells exhibiting autofluorescence, yet others intensively fluorescent. According to the author, the strong fluorescence of these cells was a result of the combination of antibodies with them. From this one could draw the conclusion that these cells were infected by cold sore viruses. According to Witmer, with the help of fluorescent antibodies it would be possible to show the presence of a cold sore virus in a routine manner in the fiber culture and in the epithelium of the cornea in cases of keratitis herpetica. Diagnostic difficulties in the use of fluorescent antibodies are connected with the lack of the presence of interpolation corpuscles in people in cases of cornea inflammation, while in rare cases of cold sore inflammation of the uvea the viruses are difficult to detect in the chamber fluid, because they are joined with the cells of the uvea.

Vozza and Balducci [24] undertook research on virus diseases of the sclera and the conjunctiva, proceeding from the assumption that fluorescent antibodies can be used in diagnostics from the point of view of the ease in which smear samples containing infected cells can be obtained. For the experiments they used a dermatotropic smallpox virus, which they introduced into the cornea of the eye of a guinea pig. In rabbits they induced keratoconjunctivitis herpetica after pre-cornea introduction of lelofilizone Z strain which singly passes to the chorion-amnion membrane of a chicken embryo. Another group of rabbits were infected through the cornea with a content of vesicles which was taken from an individual with recrudescent cold sores of the lips. After scraping off the infected cells from animals, smear tests were made up and were dyed with fluorescent serums. For dyeing the cells infected with smallpox viruses a fluorescent sheep serum was used against the rabbit serum. In the

experiments on keratoconjunctivitis herpetica a fluorescent horse antiglobulin was used against human serum. In the control tests cells not infected with viruses were dyed and infected cells were dyed with normal serums. In examining the smear tests collected in severe cases of smallpox inflammation of the cornea and uvea the authors confirmed that a significant number of cells exhibited their own fluorescence with a yellow coloration. This fluorescence was limited mainly to the cytoplasm and was grainy, while in certain cells it appeared as a shiny spot around the membrane of the cell nucleus. After coloring these cells with Giemsa's stain the presence of Guarnieri's corpuscles in them was confirmed.

In the smear samples collected from patients with trachoma, the microscopic pictures were not as equivalent as in previous experiments. The control tests in the use of uninfected cells were not always negative, since a fluorescence not peculiar to them was confirmed. In certain experiments with negative controls the cells exhibited a weak characteristic fluorescence limited to the cytoplasm, however, they did not show the presence of interpolation corpuscles in the preparations dyed by Giemsa's method. The failure to detect virus antigen material in the cells infected with trachoma viruses undoubtedly was caused by the low titer of antibodies in the marked human serums, which were used in the experiments.

As the tests by Frezotti and Berengo [12] showed, fluorescent antibodies can become a valuable aid in the detection of Toxoplasma gondii as an etiologic factor of neuro-ophthalmic infections. For this purpose the authors applied a direct reaction, using human serums with a titer in the Sabina-Feldman dye reaction of no less than 1:2000. These serums were marked with fluorescein. With the help of this technique they thoroughly examined precipitates from the cerebro-spinal fluid of 19 patients, confirming bodies similar to Toxoplasma gondii in all cases. The authors direct special attention to the fact that the correct diagnosis of toxoplasmosis of the eyeball depends on the establishment of typical changes of the bottom of the eye in connection with the additional result of the Sabina-Feldman dye reaction, however, this is an introductory diagnosis. The detection of protozoa in the eyeball would become an immediate proof, however, enucleation seldom takes place in the discussed cases. The detection of Toxoplasma gondii in the cerebro-spinal fluid with the help of fluorescent antibodies creates serious possibilities for establishing a real diagnosis in cases of symptoms on the part of the eyeball in toxoplasmosis.

From the cited list of bibliography it turns out that fluorescent antibodies are applied in three areas of

examination of the eyeball: in searching for infection factors, in particular bacteria such as Leptospira pomona and and Str. pyogenes, smallpox viruses, cold sores and trachoma and protozoa of Toxoplasma gondii, the localization of local production of antibodies and on the antigen structure of the fibers of the eye of grown individuals and in the phase of embryogenesis.

The direction of research whose purpose it is to detect and diagnose etiological, bacterial factors is encumbered with significant difficulties, which are a result of the formed antigen structure of microorganisms and their antigen relationships. Here we should once again return to the observations of Offret [20] on the properties of the chamber fluid which are harmful to bacteria and make the formation of a culture difficult. It can be assumed that this phenomenon is caused by bacteria in vivo by antibodies from the chamber fluid, which the results of his own experiments would indicate [16]. The method of sclerotic culture in the application of fluorescent antibodies creates certain bases for assuming that it will be helpful in establishing the etiology of certain forms of uvea inflammation, however, from the point of view of the negligeable number of data it is impossible to make decisive conclusions to this degree. The diagnostic application of fluorescent antibodies occurred already in the early stages of development and up to the present time not one of the immunofluorescent reactions worked out to date has completely replaced the applied methods of culture and identification of pathogenic microorganisms.

The difficulties connected with the localization and identification of viruses in the fiber structures of the eye illustrate the already cited works of Witmer [26-28] and Vozza and Balducci [24] on cold sore, smallpox and trachoma viruses. The method of obtaining antivirus serums in high titers and the improvement of the course of absorption of serums to such a degree requires further research, in order to eliminate the phenomenon of fluorescence not peculiar to it, which makes it difficult to interpret the observed pictures.

Certain successes should be noted in connection with the research on the antigen structures of the eyeball and it is possible to foster the hope that this research will be continued with greater consideration given to the production of the thirty years [23] encompassing the results of research on the fluorescence of structures of the eyeball.

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